JTUSCI

Available online at www.taibahu.edu.sa
ISSN: 1658-3655
Diab et.al. / JTUSCI 1: 24-34(2008)

Journal of Taibah University for Science

Tracing of Gram-negative antibiotic-resistant bacteria in hospitals final effluent at Al-Madinah Al- Mounwwarah

Atef M. Diab *, Idriss M. Al-Turk, Mohamed K. Ibrahim and Khalid D. Al-Zhrany

Biology Department, Faculty of Science, Taibah University, Al-Madinah Al- Mounwwarah*, KSA.*

Abstract

The final effluent for 5 hospitals (3 governmental and 2 private) was surveyed in order to study their bacterial population including, total viable bacteria (TVB) and total coliform (TC) counts / ml, macro and micro- morphology of the isolated and identification of the isolated and purified bacterial strains to the specific level confirmed using API strips and its code index matching computer program. Counts allover the study showed figures beyond the international permissible limits, ranging between $1x\ 10^3 - 1\ x\ 10^7$ cfu/ml. Identification confirmed that the bacterial population is composed of 27 species belonging to 17 genera; *Escherichia coli 1, Klebsiella pneumoniae, K. ornithinolytica, Providencia alcalifaciens, Salmonella arizonae, Enterobacter cloacae, E. saburiae, E. gergoviae, Yersinia pestis, Citrobacter frundii, Serratia marcens, Pseudomonas aeruginosa, P. putida, Flvimonas oryzihabitans, Chryseomona luteda, Stenotrophomonas maltophila, Shewanella putrifaciens,Aeromonas hydrophila, A. salmonicida, Chryseobacterium meningiosepticum, Ch. Indologenes, Pasteurella multocida, Pas. pneumotropica and Moraxella catarrhalis,* affiliated to 5 families; Enterbacteriaceae, Pseudomonadaceae, Vibrionaceae, Pasteurellaceae and Moraxellaceae. Gram negative bacterial strains dominated specially those of family Enterobacteriaceae and Pseudomonadaceae. The higher distribution patterns amongst the isolated strains, from almost all samples were for *E. coli* 1, followed by *Enterobacter cloacae* and *Chryseobacterium meningiosepticum.*. Antibiotic assay (9 antibiotics from different families) on 153 representative strains using antibiotic serial dilutions from 10 up to 100 µg/ml- to determine MIC (s), MBC (s) and the MIC/MBC indexes. All the studied strains exhibited resistance to at least 3 of the 9 tested antibiotics. Many bacterial isolates resist the whole 9 antibiotics and to a concentration of more than 100 µg/ml. The majority of the resistance strains were gram negative and the mechanism of action proved to be inhibitory.

Keywords: Antibiotic-resistance, Gram-negative bacteria, hospitals final effluent

Introduction

One tenth or more of the world's population consumes food produced through irrigation with recycled wastewater; treated, partially treated and/or untreated hospital waste effluents. Hospital waste effluents, even if it is treated, may contain pathogenic drug- resistant bacteria, which constitute the most dangerous single risk factor for dissemination of pathogenic and drug resistant organisms to the environment [1]. When a human or an animal is given a drug, from 50% to 90% of it is excreted unchanged. The remainder is excreted in the form of metabolites; chemicals produced as byproducts of the body's interaction with the drug. According to the CDC estimates, about 22000 tons of antibiotics are produced annually in the United States alone, 50% of it is dispensed to humans. Residues of up to 10 different drugs have been found in such water at concentrations totaling 6 ppb. German scientists reported that anywhere from 30 to 60 drugs can be measured in a typical water sample, if anyone takes the time to do the proper analyses. Fifty-eight percent of samples in a recent studies had at least one antibiotic present while 25% had three or more; sulfamethoxazole, trimethoprim, ciprofloxacin, ofloxacin, lincomycin, and penicillin G, [2-5].

Antibiotics may be present at levels that could not only alter the ecology of the environment but also give rise to antibiotic resistance [6]. Many drugs are also designed to be persistent, so that they can retain their chemical structure long enough to do their therapeutic work. It is well

known that the drugs enter the aquatic environment and eventually reach drinking water if they are not biodegraded or eliminated during sewage treatment. Additionally, antibiotics and disinfectants are supposed to disturb the wastewater treatment process and the microbial ecology in surface waters. Recently, pharmaceuticals have been detected in surface water, ground water and drinking water. However, only little is known about the significance of emissions from households and hospitals. Guidance on this is available in a number of publications [7-9] which cover both simple techniques, such as the simple pit latrine, ventilated pit latrine, and pour-flush latrine, and the more advanced septic tank with soakaway or the aqua-privy.

Hospital effluent with its high content of multidrug resistant enterobacteria and the presence of enteric pathogens could pose a grave problem for the community [10-12]. The occurrence of strongly selective environments for antimicrobials, such as hospitals, promotes, not only the growth of resistant bacteria, but also leads to an increase in the frequency of resistance bacterial genes and genetic elements such as plasmids.Some microorganisms important in human infection such as the Gram-negative rods, including enterobacteria such as Enterobacter sp., and other non-glucose fermentors, for example Pseudomonas aeruginosa, may persist for long periods in the environment [13-17]. Fluoroquinolones, a class of broad-spectrum antibiotics, are well known to be the leading source of a hospital wastewater's toxicity damaging DNA [18].

Unfortunately, it also means that, once they are excreted into the environment, they enter food chains and concentrate as they move upward into larger predators. Comparing the amounts of certain pharmaceuticals including some antibiotics; trimethoprim (5000 ng/l), ofloxacin (35000 ng/l) and penicillin G (5000 ng/l), those drain from different point sources of pollution as hospitals, proved the many times more of these antibiotics drained from hospitals than from other sources, [4, 19, 20].

Over evolutionary time, bacteria challenged into the 21st century and the most recent knowledge about the mechanisms of resistance action of antibiotics such as tetracyclines, kanamycin and aminoglycoside postulated the active extrusion of structurally unrelated drugs from the cell [21-24]. However, many regulatory systems are ill-adapted for detecting the presence of toxic pump substrates and instead are likely to respond to alternative signals related to unidentified physiological roles of the transporter [25]. The possibility of persistence and transmission of drug-resistant gram negative bacteria from hospitals wastewater to the environment and community, especially in relation to a sewage treatment process based on waste water recycling, is a scientific fact .

Now the massive uncontrolled use of antimicrobial agents, antibiotics in particular, was and still one of major threatens that man enrolled in his “Agenda 21” [26].

The present research work aimed at revealing the possibility of contamination of the environment with Gram-negative antibiotic-resistant bacteria, coming out from hospitals drains in Al-Madinah Al- Mounwwarah.

This is a part of the research project sponsored by Taibah University.

Material s& Methods

Samples Collection

Samples were collected from sewers receiving final effluents from five hospitals.

Sampling sites were selected as to cover both private and governmental hospital sectors, those specialized in certain community groups and/or community diseases

Sterilized, screw-capped, pyrogen-free 250 ml glass conical flasks, were used to collect samples. Transportation regime to the laboratory within 2 hours in ice jackets with ice bags, was followed. This was conducted with accordance with the EPA – approved – Quality Assurance Project Plan (2003). Each site was sampled five times on bimonthly basis, during the period from August 2005 to June 2006.

Continuous shaking in an orbital shaker for 5 min. at 200 rpm proceeded just before serial dilutions up to 10^{-4} was achieved in standard saline solution. Standard plate count method on plate count agar medium (Scharlau) was then adopted, inoculating 1 ml of each dilution onto 9 cm sterile plastic Petri dish, for total viable counts of bacteria. Running of a parallel set using MacConkey`s agar medium (Scharlau) was achieved for total coliform counts. Colony-forming units (cfu/ml) were enumerated using electric counter after incubation of the inoculated plates at 37º C for 24 and 48 hours [27].

Antibiotic-Resistance Studies

Serial dilutions; 10, 30, 50, 70 and 100 µg/ml, were prepared from 9 antibiotics namely; bacitracin, chloramephincol, erythromycin, impenim, penicillin G, rifampicin, streptomycin, tetracycline and vancomycin, comprising the most widely- spread and commonly prescribed by physicians. They were also chosen as to represent different antibiotic families. Sterile saline solution, (0.85 % NaCl) 5 ml aliquots were inoculated with 200 µl of bacterial suspensions adjusted to a density of approx. 10^{7} to 10^{8} using basic 0.5 McFarland standard solution, [28]. Readings of the MIC (s) were recorded after 24 & 48 hours of incubation at 37º C. Three replica of nutrient agar plates were inoculated with 100 µl each from the tubes of MIC test starting from one tube before the MIC one, for the isolates showed resistance.

Identification of Bacterial Isolates

Bacterial identification was based on colony morphology on nutrient agar culture medium (Oxoid), bacterioscopy of Gram-stained bacterial streaks, motility, standard oxidation, oxidase production, glucose fermentation, urease activity, H2S production (from sulfer-containing aminoacids), indole from tryptophan, use of citrate and decarboxylation of lysine, arginine and ornithine. Bacterial inocula from purified 24 hours freshly-cultured single colonies were adjusted to a growth density of approx. 10^{7} to 10^{8} using basic 0.5 McFarland standard solution , [28, 29]. API 20E strips, Api Web computer program (from BioMerieux, Inc.) and API protocol adopted from the science advisory board, helped in identifying the bacterial isolates (153) up to 95% confidence ID results.

Statistical Analyses

Categorical variables were presented in frequency, percentage, mean ± SD and ratios, using excel and prism 4, statistics & graphic computer programs. API web computer program was applied for the identification of bacterial isolates.

Results & Discussion

Counting results

King Fahd hospital wastewater bacteriological analysis showed fluctuated mean counts for the total viable bacteria (TVB), between 2×10^{2} and 308×10^{4} cfu/ml and for the total coliforms (TC) 1.4×10^{2} and 91×10^{4} cfu/ml. The gram negative bacteria presented up to 270×10^{4}, while gram positive share was only 70×10^{2} cfu/ml; detailed from 5 successive samples (Fig. 1).

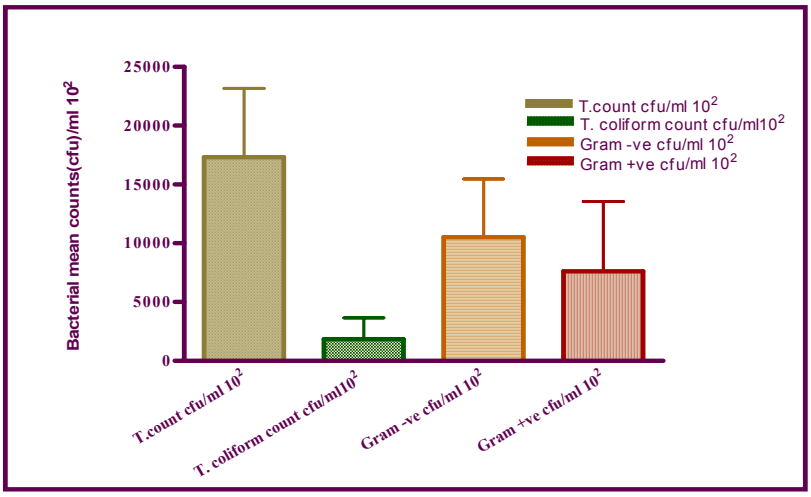

Fig. 1. Total viable bacteria (TVB), total coliform (TC), Gram –ve and Gram +ve mean counts x 10²(cfu)/ml, in the final mixed wastewater from King Fahd hospital.

Ohod hospital wastewater bacteriological analysis showed quietly different figures being 2 x 10^2 and 382 x 10^3 cfu/ml for TVB, 0.0 and 12.6 x 10^2 fu/ml for TC, 2 x 10^2 and 382 x 10^3 cfu/ml for gram negative bacteria and 0.0 to 98 x 10^2 cfu/ml for gram-positive (Fig. 2).

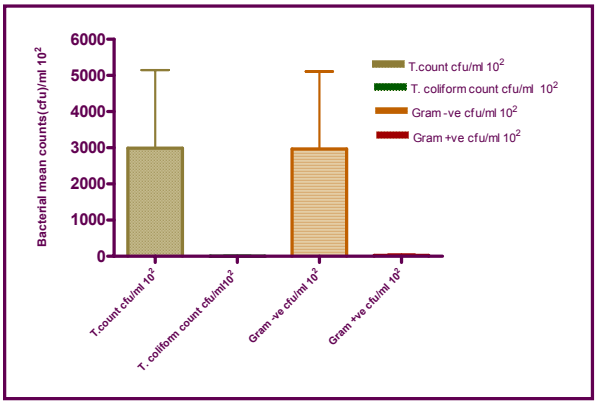

Fig. 2. Total viable bacteria (TVB), total coliform (TC), Gram –ve and Gram +ve mean counts x 10² (cfu)/ml, in the final mixed wastewater from Ohod hospital.

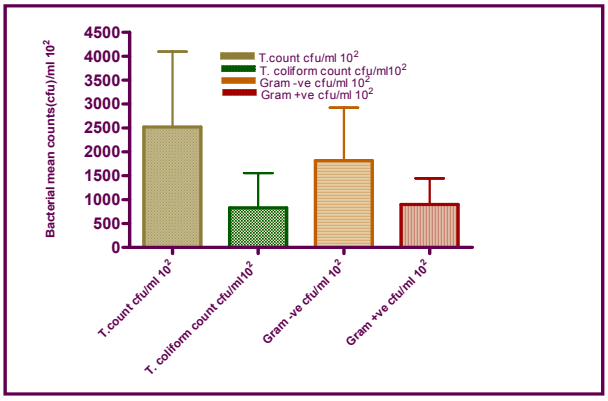

Fig. 3. Total viable bacteria (TVB), total coliform (TC), Gram –ve and Gram +ve mean counts x 10² (cfu)/ml, in the final mixed wastewater from Women and Maternity hospital.

Women and Maternity hospital wastewater bacteriological analysis showed almost the same trend of fluctuation being 37.7 x 10^2 and 454 x 10^3 cfu/ml (TVB), 5.2 x 10 2 and 373 x 10 3 cfu/ml cfu/ml (TC), 0.0 and 372 x 10^3 cfu/ml gram negative and 0.0 to 181 x 10^3 cfu/ml gram positive, (Fig. 3).

Saudi German hospital wastewater bacteriological analysis showed counts ranged between a minimum of 84.7 x 10^2 and a maximum of 584 x 10^3 cfu/ml (TVB), from 19.6 x 10^2 to 92.6 x 10^2 cfu/ml (TC), from 80 x 10^2 to 497 x 10^2 cfu/ml gram negative and from 4.3 x 10^2 to 201 x 10^2 cfu/ml gram positive, (Fig. 4).

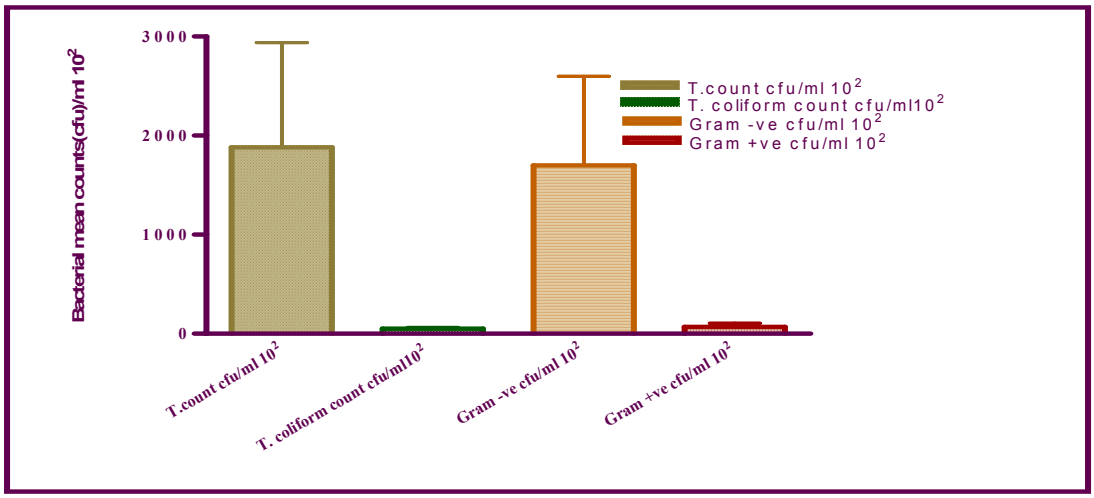

Fig. 4. Total viable bacteria (TVB), total coliform (TC), Gram –ve and Gram +ve mean counts x 10^2 (cfu)/ml, in the final mixed wastewater from Saudi german hospital

Al-Moasah hospital wastewater bacteriological analysis showed counts ranged between a minimum of 360 x 10^2 and a maximum of 293 x 10^4 cfu/ml (TVB), from 23 x 10^2 to 757 x 10^3 cfu/ml (TC), 288 x 10^2 to 260 x 10^4 cfu/ml gram negative and from 0.0 to 325 x 10^3 cfu/ml gram positive (Fig. 5).

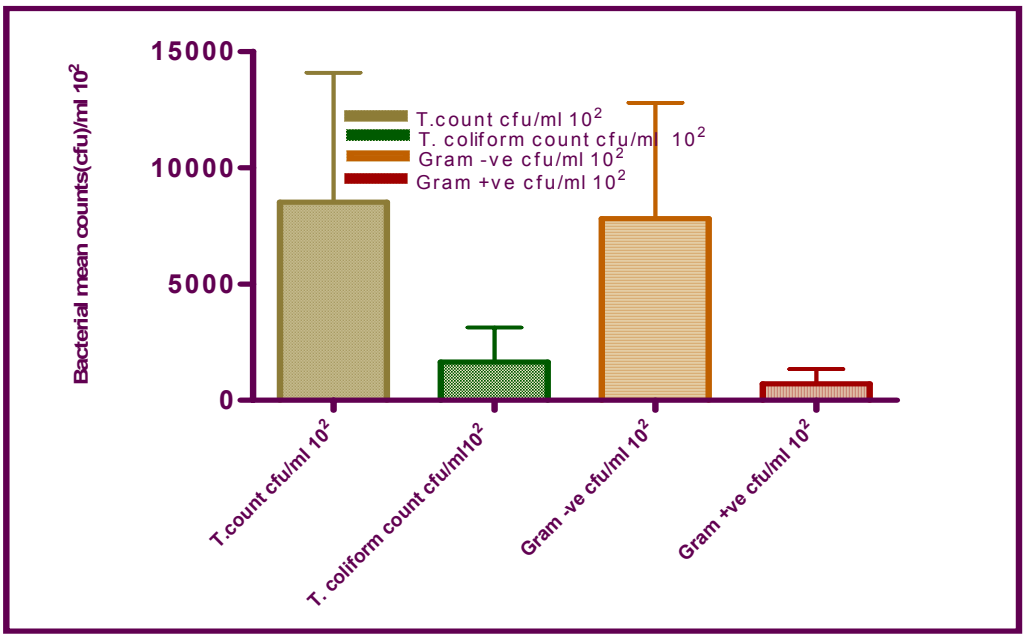

Fig. 5. Total viable bacteria (TVB), total coliform (TC), Gram –ve and Gram +ve mean counts x 10^2 (cfu)/ml, in the final mixed wastewater from Al-Moasah hospital.

Counts for all bacterial groups investigated at all the studied sites in the five hospitals indicated high levels of pollution, even if the hospital has a treatment facility (Saudi German) or not as the rest of the hospitals under investigation. Safe discharge of such wastewater category should not exceed 100 cfu/ml for (TVB), 30 cfu/ml for (TC) according to the EPA Guidelines (2003). The effluent input showed 55% of the 8.6×10^6/ml bacteria as coliforms and *E. coli* which was a typical of fecal flora. The prevalence of multi-drug-resistant (MDR) coliforms was 0.26% [11].

High contamination in all departments he investigated specially I.C.U., Ob. and Lab. expressed in cfu/ml, in King Fahd, Ohod and Al-Anasar hospitals was found during 2004 [30]. From this point it is clear that all the wastewater qualities discharged from the studied localities are real source of pollution that is directly introduced to the environment.

Comparing total viable bacterial counts recorded for the five hospitals revealed that the highest polluted levels were at Al-Moasah hospital, followed by King Fahd hospital, Women and Maternity, Ohod and the lowest pollution levels were detected in Saudi German, in descending order, Fig. 6. Total coliforms counts profile was of a different order being; King Fahd hospital, Al-Moasah hospital, Women and Maternity hospital, Saudi German hospital and lastly Ohod hospital, Fig. 7, this is quite in line with the findings of a similar study [30].

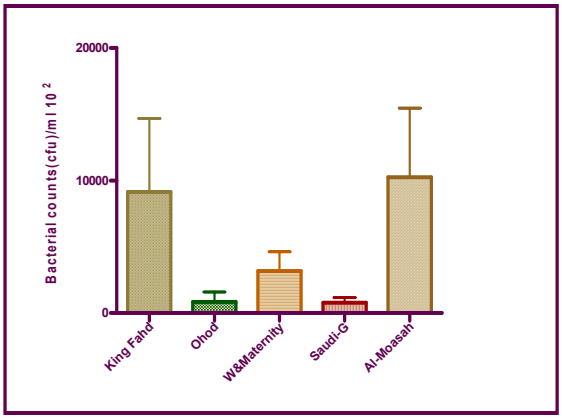

Fig. 6. Total viable bacterial (TVB) mean counts x 10^2 (cfu)/ml, in the final mixe wastewater from King Fahd, Ohod, Women and Maternity Saudi German and Al-Moasah hospitals.

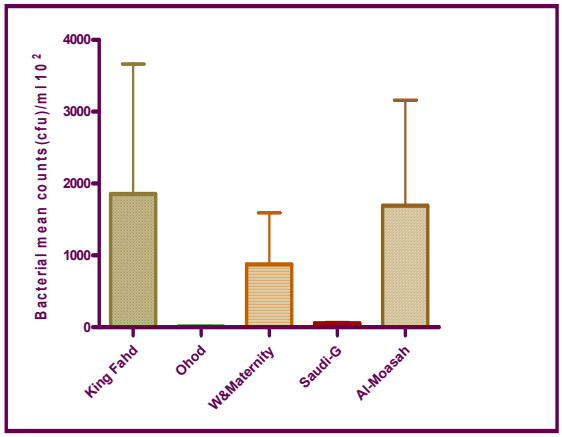

Fig. 7. Total coliform bacterial (TC) mean counts x 10^2 (cfu)/ml, in the final mixedwastewater from King Fahd, Ohod, Women and Maternity, Saudi German and Al-Moasah hospitals.

Identification

The overall distribution of isolated bacteria in the whole study showed a superior dominance of gram negative rods (70%), then gram positive rods (27%), gram positive cocci (2%) and gram negative cocci (1%), Fig. 8. and reports [10, 16, 17, 31-37]. Other important

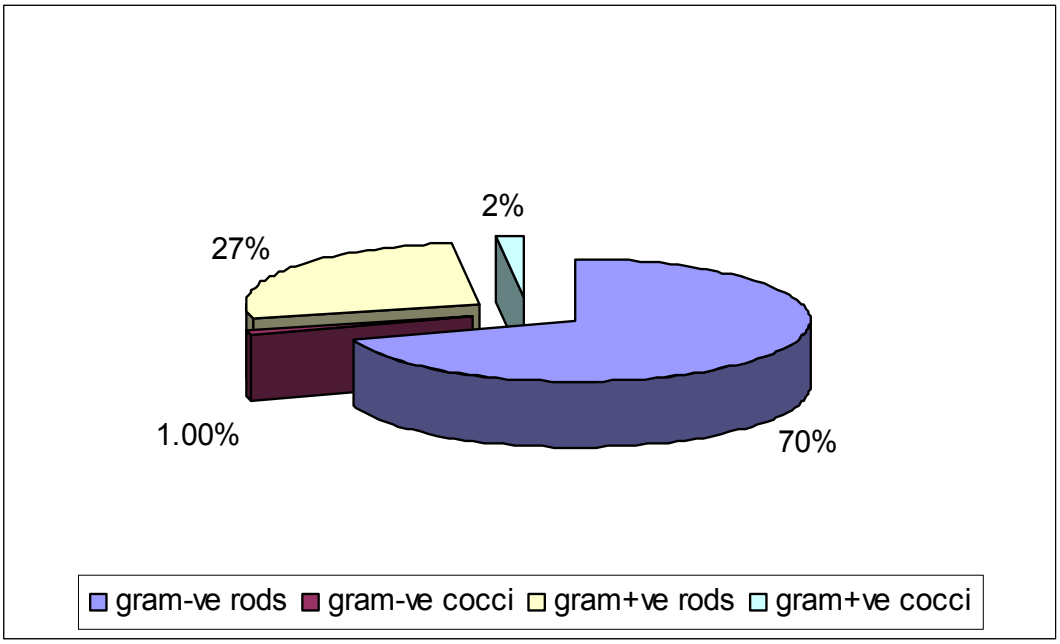

Fig. 8. The overall distribution of bacteria from the studied hospitals in this study, as percentage of Gram +ve and Gram –ve rods and cocci.

A total of 153 gram negative isolates (70 % of the studied bacterial population), were identified to the specific level using API 20 E strips and API WEB. Program. Species (27) inhabiting the hospitals wastewaters environment, were found to belong to 17 genera affiliated to 5 families. Family: Enterobacteriaceae with 8 genera and 11 species occupied the first position followed by Family: Pseudomonadaceae with 4 genera and 5 species, Family: Vibrionaceae with 3 genera and 5 species, Family: Pasteurellaceae with its only genus and two species and finally Family: Moraxellaceae with its only genus and one species. The relative dominance of Enterobacteriaceae members, *Escherichia coli* 1 in particular at all the studied sites is in line with most of the published papers presence of bacteria like *Escherichia coli* 1 and *Klebsiella pneumoniae*, amongst the 24 isolated species, either as human pathogens and/or opportunistic human pathogens [15, 36, 37, 39]. The most repeatedly isolated species was *E. coli* , which came in the first place being a principal member in all samples at all hospitals all the time period of study. *Enterobacter cloacae and Chryseobacterium meningiosepticum* came in the second place, followed by *Klebsiella pneumoniae* and other 7 species, before the last 16 species those shared equally the same distribution.

Collectively, the results suggested that the profiles for the observed antibiotic resistance in all sites arose from taxonomic differences in the culturable bacterial population at the generic or

subgeneric level. It is considerable that *Enterobacter* sp. and *Stenotrophomonas maltophilia*, may be considered potential indicator organisms to assess microbial tolerance in various compartments of the aquatic environment.

Antibiotic – resistance

The antibiotic – resistance profiles of representative (one isolate) for each of the identified species, were as recorded in Table (1). Most results were clear after 24 hours, but some bacterial isolates showed slow-growing trend, so results were recognized after 48 hours. This is may be because of the extreme nature of ecosystem they isolated from. This, of course, would take a little more time of relative recovery under the laboratory conditions. One way or another the same interpretation was adopted by other researchers [19, 21, 25]. The majority of them were resistant to at least 3 antibiotics with MIC (s) ranged between 50 and 100 µg/ml, while resisting all of the 9 antibiotics was recorded for 12 species to more than 100 µg/ml. It is important here to declare that 100% of the examined members belong to F. Enterobacteriaceae were resistant to the 9 antibiotics (Fig. 9).

In a similar study carried out in southern Austria, a total of 767 *E. coli* isolates were tested regarding their resistance to 24 different antibiotics. The highest resistance rates were found in E. coli strains of a sewage treatment plant which treats not only municipal sewage but also sewage from a hospital [32, 40]. It was also concluded that: E. coli was the main carrier of antimicrobial resistance in fecal flora; resistance in other species was rare in the absence of antimicrobial selection [32]. *Aeromonas* sp., *Chryseobacterium* sp., *Pasteurella* sp. and *Pseudomonas* sp. showed also the same antibiotic – resistance profiles as those of the Enterobacteriaceae members. Hospital heterotrophs displayed a higher frequency (84%) of ampicillin (Amp) tolerance compared to the heterotrophs from the freshwater fishfarm site (22%). Results indicated that this effect was linked to the predominance of intrinsically ampicillin-resistant strains over representatives of Acinetobacter and Escherichia coli within the hospital strain set. Tolerant strains for hospital aeromonads were (43%) in comparison with the fishfarm aeromonads (8%) [41].

Results of MIC/MBC indexes revealed that 87% of the isolates were negatively affected with antibiotics; i.e. the action of such antibiotics on them was merely bacteriostatic and not bactericidal. This may explain the irresponsiveness and/or the elevated rate of disease recurrence in the last ten years, this finding was also documented by others [42, 43]**.** The inhibited or attenuated treated bacterium with such bacteriostatic antibiotic gives the microbe the chance of recovery just after the concentration of the antibiotic in the body is getting lower than its MIC limits [15, 16, 33]. Since no susceptibility breakpoints were available for most of the antibiotics discussed, an alternative approach to the interpretation of MICs was presented [2]**.** Strains from contemporary populations of *Escherichia coli* and *Salmonella enterica* displayed high MICs to at least one of the antibiotics were detected in many other studies [31].

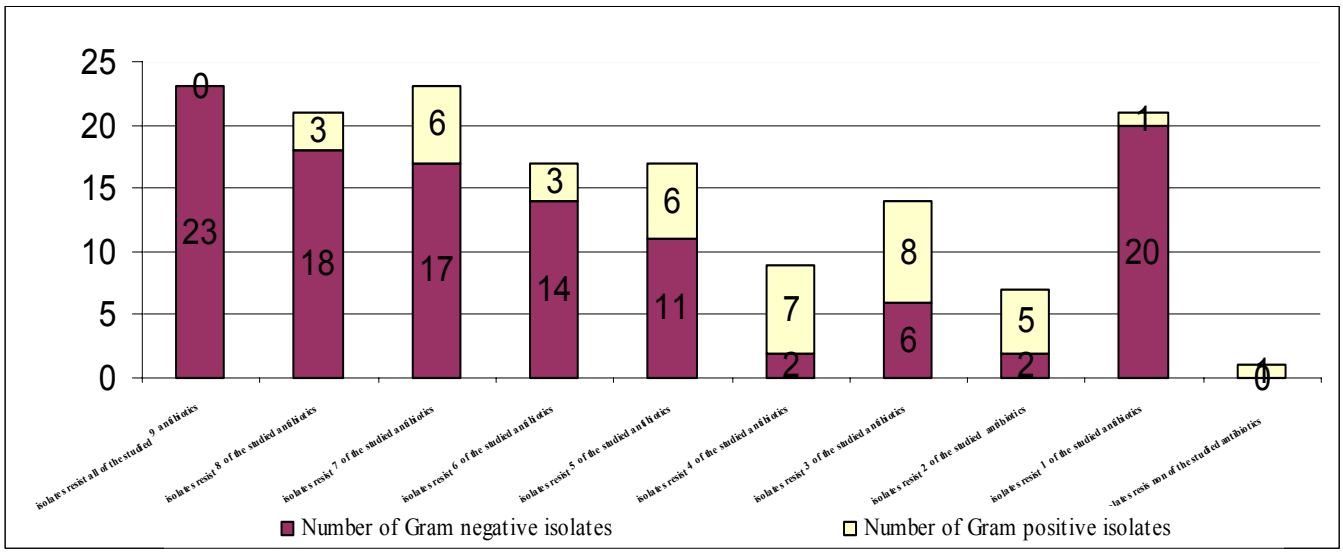

Fig. 9. Distribution of Gram –ve bacterial isolates based upon the number of antibiotics they resist out of 9 screened antibiotics

Table 1. Shows 27 representatives of all of the isolated and identified bacterial species during the 10 months study period, where (-) sensitive to down to 10 µg/ml, (+) resistant to up to 50 µg/ml, (++) up to 70 µg/ml and (+++) resistant to more than 100 µg/ml.

Bacterial species	Bacitra.	Chlor	Eryth.	Imipen.	Penici. G	Rifam.	Strept	Tetracy.	Vanco.
***E. coli* 1 (99)**	+++	+	+++	+++	+++	+++	+	++	+++
***Entero. Cloacae* (51)**	+++	+	+++	+++	+++	++	+	+	+++
***Entero. Saburiae* (104)**	+++	-	+++	+++	+++	+++	+++	-	-
***Entero. Gergoviae* (55)**	+++	+	+	+++	+++	-	+++	-	+++
***Citro. freundii* (52)**	+++	+	+	+++	+++	+++	+++	+++	+++
***Kleb. Pneumo* . (76)**	+++	+	++	+++	+++	+	+++	+	+++
***Kleb. Ornithinolytica* (77)**	+++	-	++	+++	+++	+	+	+	+++
***Moraxella sp.* (123)**	+++	-	+	-	-	-	++	++	+++
***Serratia ficaria* (97)**	+++	+++	+++	+++	+++	+++	+++	+++	+++
***Serratia odorifera* 1 (101)**	+++	-	++	+++	+++	++	-	+	+++
***Serratia marcens* (6)**	+++	++	+++	+++	+++	++	-	-	+++
***Salmonella arizonae* (45)**	+++	+	+	+++	+++	+	-	-	+++
***Yersinia pestis* (75)**	+++	-	++	+++	+++	++	-	-	+++
***Pseudomonas putida* (3)**	+++	+	+	++	+++	++	++	++	+++
***Pseudomonas aeruginosa* (1)**	+++	++	-	++	+++	-	++	-	+++
***Aeromonas salmonicida* (67)**	+++	++	+++	+++	+++	+++	+++	+++	+++
***Aerommonas hydrophila* (19)**	++	++	+++	+++	+++	++	+	+	+++
***Flavimonas oryzihabitans* (105)**	+++	++	-	++	+++	-	+++	-	-
***Chryseomonas luteda* (89)**	+++	+++	+++	+++	+++	+++	+++	+++	+++
***Pasteurella multocida* (82)**	+++	+	+	-	++	-	-	-	-
***Pasteurella pneumotropica* (27)**	+++	+	+++	+++	+++	+	+++	++	++
***Stenotrophomonas maltophilia* (17)**	+++	+	+	+++	+	+	-	++	+++
***Chryseo.. meningioseptecum* (66)**	+++	+	+++	+++	+++	+++	+++	+++	+++
***Chryseo. Indologenes* (136)**	+++	-	-	+	-	-	-	-	-
***Shewanella putrifaciens* (42)**	+++	-	+	+	+++	-	+	-	+++
***Providencia alcalfaciens* (33)**	+++	-	-	++	+	-	++	++	-
***Ochrobacterium anthropi* (122)**	+++	-	-	-	-	+	-	-	+++

References

[1] Rahman M, Huys G, Rahman M, Albert J, Kuhn I and Mollby R, (2005). App. Environ. Microbiol,. **58**: 29-37.

[2] Butaye, P.; Devriese, L.A. and Haesebrouck, F. (2003). Clinical Microbiology Reviews, Vol. **16**: 175-188.

[3] Gilbert, P. and McBain, A.J. (2003). Clinical Microbiology Reviews, **16**: 189-208.

[4] Brown K. D., (2004). *Pharmaceutically active compounds in residential and hospital effluent, municipal wastewater, and the Rio Grande in Albuquerque, New Mexico. www.unm.edu/~wrp/*

[5] Brown, K. D., Kulis, J., Thomson, B., Chapman, H. and Mawhinney, D. B., (2005). Brazilian J. of Microbiol , **31**,: 112-117.

[6] Janet Raloff, (1998). "*Drugged Waters*," Science News,**153**: 187-189.

[7] Franceys R, Pickford J, Reed R (1992). *A guide to the development of on-site sanitation.* Geneva, World Health Organization.

[8] WHO, (1999). *Safe Management of Wastes from Health Care Activities*. 10. Collection and disposal of wastewater 10. 3. 1. Pharmaceuticals

[9] Mara D (1996). Low-cost urban sanitation. Chichester, England, Wiley.

[10] Cabrera, R.; Ruiz, J.; Marco, F.; Oliveira, I.; Arroyo, M.; Aladueña, A.; Usera, M.A.; De Anta, M.T.J.; Gascón, J. and Vila, J. (2004). Antimicrobial Agents and Chemotherapy, **48**: 3934-3939.

[11] Chitnis V, Chitnis S, Vaidya K, Ravikant S, Patil S andChitnis DS, (2004). Water Research, **38**: 441-447.

[12] Somwang, D., Wichai, W., Susan, A. and Duangporn, J., (2005). J. Med. Assoc. Thai. Vol. **88**: 59- 64.

[13] Bradford, P.A. (2001). Microbiology Reviews, Vol. **14**: 933-951.

[14] Frederico de Meirelles-Pereira; Angela de Meirelles Santos Pereira; Márcio Cataldo Gomes da Silva; Verônica Dias Gonçalves; et al. (2002). Brazilian J. of Microbiol. **33**: 193-205.

[15] Miller, K.; O'Neill, A.J. and Chopra, I. (2004).. Antimicrobial Agents and Chemotherapy, **48**: 23-29.

[16] Wiuff, C.; Zappala, R.M.; Regoes,, R.R.; Garner, K.N.; Baquero, F. and Levin, B.R. (2005). Antimicrobial Agents and Chemotherapy, **49**: 1483-1494.

[17] Xu, M.; Zhou, Y.N.; Goldstein, B.P. and Jin, D.J. (2005). Journal of Bacteriology, **187**: 2783-2792.

[18] Hartmann, A. et al, (1997). Society of Environmental Toxicol. and Chemistry. San Francisco. USA.

[19] Merz, L.R.; Warren, D.K.; Kollef, M.H. and Fraser, V.J. (2004). Antimicrobial Agents and Chemotherapy, **48**: 2861-2865.

[20] Perreten, V.; Fawer, L.V.; Slickers, P.; Ehricht, R.; Kuhnert, P. and Joachim Frey, J. (2005).. Journal of Clinical Microbiology, **43**: 2291-2302.

[21] Putman, M.; van Veen, H.W. and Konings, W.N. (2000). Microbiology and Molecular Biology Reviews, **64**: 672-693.

[22] Chopra, I. and Roberts, M. (2001). Microbiology and Molecular Biology Reviews, **65**: 232-260.

[23] Koch, A.L. (2003). Clinical Microbiology Reviews, **16**: 673-687.

[24] Vakulenko, S.B. and Mobashery, S. (2003). Clinical Microbiology Reviews, Vol. 16, No. 3, p. 430-450.

[25] Grkovic, S.; Brown, M.H. and Skurray, R.A. (2002). R Microbiology and Molecular Biology Reviews, **66**: 671-701.

[26] UNIDO, the biotechnology management service, (1994). Vienna International Center.

[27] APHA (1992). Standard Methods for the Examination of Water and Wastewater. 18 ^{ed}. (Eds Greenberg, Connors and Jenkins), American Public Health Association, Washington, D.C.

[28] NCCLS, (1990). Quality assurance for commercially prepared microbiological culture media. Approved standard document M22-A **10**, No. 14.

[29] Murray, P. A., (1999). Manual of Clinical Microbiology, 7th ed.

[30] Al-Turk, I. M., (2004). Al-Azhar Bull. Sci. **15**: 1-7.

[31] Houndt, T. and Ochman, H. (2000). Applied and Environmental Microbiology, **66**: 5406-5409.

[32] Österblad, M.; Hakanen, A.; Manninen, R.; Leistevuo, T.; Peltonen, R.; Meurman, O.; Huovinen, P. and Kotilainen, P. (2000). Antimicrobial Agents and Chemotherapy, **44**: 1479-1484.

[33] Winokur, P.L.; Brueggemann, A.; DeSalvo, D.L.; Hoffmann, L.; Apley, M.D.; Uhlenhopp, E.K.; Pfaller, M.A. and Doern, G.V. (2000). Antimicrobial Agents and Chemotherapy, **44**: 2777-2783.

[34] Randall, L.P. and Woodward, M.J. (2001). Applied and Environmental Microbiology, **67**: 1190-1197.

[35] Grohmann, E.; Muth, G. and Espinosa, M. (2003). Microbiology and Molecular Biology Reviews, **67**: 277-301.

[36] Nishino, K. and Yamaguchi, A. (2004). Journal of Bacteriology, **186**: 1423-1429.

[37] Koutsolioutsou, A.; Llopis, S.P. and Demple, B. (2005). Antimicrobial Agents and Chemotherapy, **49**: 2746-2752.

[38] Steger-Hartmann T. and others, (1997). Ecotoxicol. and Environ. Safty, **36**: 174-179.

[39] Baucheron, S.; Tyler, S.; Boyd, D.; Mulvey, M.R.; Dancla, E.C. and Cloeckaert, A. (2004). Antimicrobial Agents and Chemotherapy, **48**: 3729-3735.

[40] Replidyne, J P Morgan Healthcare conference (2006). an acrobat document at a net site. www. Replidyne.com, www. AlphaRx.com

[41] Diab, A. M., Abdelrahman, A. A. and Abd El-Latif, H. K., (2004). Egyptian J. Microbiol., **9**: 345-356.

[42] Brooun, A.; Liu, S. and Lewis, K. (2000). Antimicrobial Agents and Chemotherapy, **44**: 640-646.

[43] Lepper, P.M.; Grusa, E.; Reichl, H.; Högel, J. and Trautmann, M. (2002). Antimicrobial Agents and Chemotherapy, **46**: 2920-2925.